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Exceptional preservation of reproductive organs and giant sperm in Cretaceous ostracods

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The bivalved crustacean ostracods have the richest fossil record of any arthropod group and display complex reproductive strategies contributing to their evolutionary success. Sexual reproduction involving giant sperm, shared by three superfamilies of living ostracod crustaceans, is among the most fascinating behaviours. However, the origin and evolution of this reproductive mechanism has remained largely unexplored because fossil preservation of such features is extremely rare. Here, we report exceptionally preserved ostracods with soft parts (appendages and reproductive organs) in a single piece of mid-Cretaceous Kachin amber (approximately 100 Myr old). The ostracod assemblage is composed of 39 individuals. Thirty-one individuals belong to a new species and genus, *Myanmarocypris hui* gen. et sp. nov., exhibiting an ontogenetic sequence from juveniles to adults (male and female). Seven individuals are assigned to *Thalassocypris* sp. (Cypridoidea, Candonidae, Paracypridinae) and one to *Sanyuania* sp. (Cytheroidea, Loxoconchidae). Our micro-CT reconstruction provides direct evidence of the male clasper, sperm pumps (Zenker organs), hemipenes, eggs and female seminal receptacles with giant sperm. Our results reveal that the reproduction behavioural repertoire, which is associated with considerable morphological adaptations, has remained unchanged over at least 100 million years—a paramount example of evolutionary stasis. These results also double the age of the oldest unequivocal fossil animal sperm. This discovery highlights the capacity of amber to document invertebrate soft parts that are rarely recorded by other depositional environments.

1. Introduction

Ostracod microcrustaceans are ecologically diverse in the present day, with representatives in nearly all known types of marine and nonmarine waterbodies and even some (semi-)terrestrial habitats [1]. They are the most abundant fossil arthropods with a stratigraphic range dating back to the Ordovician [1]. Although the vast majority of fossil ostracods are represented only by calcified shells, rare examples with preserved appendages and other soft parts provide exceptional insights into their evolutionary history, including aspects of their reproductive strategies such as brood care, sex and parthenogenesis (e.g. [2–6]). Sexual reproduction involving giant sperm is a unique reproductive strategy shared by three superfamilies of living ostracods (the entire suborder Cypridocopina, including most nonmarine living ostracods) [5]. However, the evolution of such a complex reproductive mechanism has remained largely unexplored because of the lack of fossil reproductive organs and especially the direct evidence of giant sperm. Hitherto, giant sperm have been reported in 17-million-year-old ostracods, but only traces of male sperm pumps have been found in older material [4,5]. Here we report giant sperm in an exceptional cypridoidean ostracod assemblage with preserved soft parts (adult males and females, as well as juveniles) found in a single piece of Cretaceous amber (approximately 100 Myr old) from Myanmar (figure 1) [7].

2. Systematic palaeontology

Our reconstructions of these specimens using three-dimensional X-ray microscopy shed new light on the evolution of sexual reproduction in Ostracoda and, in particular, the antiquity of giant sperm. Thirty-one of the 39 specimens belong to a single new taxon, classified as Arthropoda, Crustacea, Ostracoda, Podocopa, Podocopida, Cypridocopina, Cypridoidea, Candonidae, Paracypridinae and described herein as *Myanmarocypris hui* gen. et sp. nov.

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Etymology: Myanmar (the country of origin) + Cypris; hui (after the collector of the amber piece, Mr Cheng HU).

Type material: Holotype, adult male (BA19005-1, figure 2a,d and electronic supplementary material, movie S1). Paratypes, adult female (BA19005-2, figure 2b,c,e,f and electronic supplementary material, movie S2) and three juveniles (BA19005-3, figure 2g,k; BA19005-4, figure 2h,l; BA19005-5, figure 2i,m); all curated in the Lingpoge Amber Museum (Shanghai).

Type locality and stratigraphy: Hukawng Valley, Kachin Province, Myanmar; upper Albian–lower Cenomanian [7].

Diagnosis (for genus and species, since genus is monospecific): Candonid with a subtriangular carapace, thick-shelled with strong reticulate external ornament. Adult valves each with a prominent external anterolateral node; internally an anteroventral submarginal tooth in the left valve (LV) with the corresponding socket in the right valve, (RV), and two posteroventral submarginal teeth in the LV.

Description: Adult carapace small (length 0.59 mm, height 0.36 mm), subtriangular in lateral view; sexually dimorphic, female with higher dorsal peak than male. In both sexes, each valve externally strongly reticulate with prominent transverse muri, conspicuous normal pores, a prominent anterolateral node and a smooth anterodorsal eye-spot. LV overlaps RV on dorsal and ventral margins. Hinge adont. Adults with a well-developed calcified inner lamella, broader anteriorly than posteriorly, bearing a rounded anterolateral tooth in LV and corresponding socket in RV, plus a pair of small teeth posteroventrally in LV. Antennula seven-segmented, antenna five-segmented with well-developed natatory setae. Terminal segment of mandible palp rectangular, terminal segment of maxillula palp almost square. Fifth limb with a six-rayed branchial plate and sexually dimorphic, male endopodite forming a two-segmented clasper. Uropodal rami with well-developed claws. Male Zenker organ with

five whorls of spines and sperm duct. Male, female and juveniles with similar carapace and soft parts, showing similar ontogenetic sequence to living taxa from juveniles to adults (figure 2).

Remarks: By virtue of its overall appendage characteristics, and particularly its Zenker organs, which have five whorls of spines, *Myanmarcypris hui* is assigned to the Candonidae, a cypridoidean family with a fossil record extending back to the mid-Jurassic approximately 170 Mya [8]. It shows close affinities with the extant candonid genus *Renaudocypris* McKenzie, with which it shares features of carapace morphology including subtriangular shape, relatively thick valves, LV > RV overlap, and the internal anteroventral tooth and socket as well as essentially similar appendage morphology. Species of *Renaudocypris* have more subdued (pitted) ornamentation than *M. hui* and have neither anterolateral nodes nor a pair of internal posteroventral teeth in the LV. Living species of *Renaudocypris* are euryhaline (oligohaline to fully marine waters), and some inhabit coral sands in the surf zone [9–11]; robust, ornamented shells and the anteroventral tooth and socket of *M. hui* may be adaptations to life in such a high-energy environment.

In addition to 31 specimens of *M. hui*, the assemblage also contains seven specimens assigned to *Thalassocypris* sp. (Cypridoidea, Candonidae, Paracypridinae) (figure 2o–r) and one to *Sanyuania* sp. (Cytheroidea, Loxoconchidae) (figure 2j,n); both genera have living representatives in marginal marine to nonmarine environments. We conclude from this evidence that the amber ostracod assemblage inhabited a marine coastal environment with reduced salinity, perhaps in an estuary, lagoon or embayment, an interpretation that is consistent with other palaeoenvironmental evidence [7].

3. Discussion

(a) Exceptional preservation of reproductive organs

The adult female of *M. hui* contains, in the posterior half of the body, four eggs (approx. 50 µm in diameter; figures 3b,g and 4c), which have similar morphology to the living counterparts (figure 4d) and a pair of seminal receptacles (figure 3b,f), here represented by a bilobate mass of curled filaments, fossil giant sperm (figures 3f and 4g,k). The receptacles' position slightly behind the middle of the body matches that in the living counterparts [12]. The spermatozoa of the ostracod superfamily Cypridoidea are thin, twisted, aflagellate but filiform cells (figure 4h,l), which can be interpreted as extremely elongated sperm-heads with a nucleus derivate extending throughout the cell [12]. The individual spermatozoon length often exceeds the body length of the ostracod itself [13]. The difficulty of following individual sperm in the tangled mass makes precise length measurements impossible, but a minimum length of 200 µm can be estimated, and their arrangement in the receptacles suggests even greater lengths. Their appearance as single filaments (not as a single agglutinated cell mass) demonstrates original external robustness and rigidity, which is a hallmark of ostracod giant sperm because they are equipped with two extracellular coats [12], one of which is very stable against decay and enhances their preservation potential (e.g. [14,15]).

The adult male of *M. hui* possesses paired Zenker organs (highly specialized muscular pumps), hemipenes and fifth limb claspers (figure 3a,c–e). Each Zenker organ, situated in the posterodorsal part of the body, consists of a tube with five whorls of sclerotized spines (figures 3d and 4e). Their position, morphology and inferred function are in accordance with the knowledge of living counterparts (figure 4a,b,e,f,i,j). Together with the fifth limb claspers and the voluminous pair of hemipenes, these features indicate a close similarity of mating behaviour between the mid-Cretaceous and living members of this group. For copulation (figure 5), cypridoidean males use their sexually dimorphic fifth limbs, which have hook-like endopods (claspers, figure 4b), to grasp a female while introducing their hemipenes into the female's paired vaginas. The male's pair of Zenker organs then transfer the exceptionally long but immotile sperm via the male hemipenes into the female [16,17]. The Zenker organ is readily identified in extant cypridoidean ostracods as a large, spiny,

sclerotized part of the deferent sperm duct. Muscle fibres alongside the organ connect the numerous spines, which are often arranged in a number of whorls that are taxonomically characteristic at family level [18]. Once in the female, the sperm are pushed up the two long sperm canals, each ending in a sac-like seminal receptacle for sperm storage; here, they finally become motile, arrange themselves into a more organized assemblage and fertilize eggs during the process of oviposition [12]. The fact that the seminal receptacles of the female are in an expanded state due to being filled with sperm indicates that successful copulation had taken place shortly before the animals became entrapped in the amber.

Sexually selected features reflect the conflict of interests caused by sexual reproduction, resulting in an 'arms race' between the sexes for advantages in mating success and mate choice [19,20]. For ostracods, it has been posited that the differentiation of a part of the male fifth limb into a clasper is an example of such an arms race [17]. The presence of clasping organs in our amber ostracods shows that this particular arms race also began earlier than 100 Ma.

(b) Exceptional preservation of the earliest animal sperm

Extraordinarily long spermatozoa occur in a wide range of animal taxa, particularly in arthropods, but also in nonarthropod groups (e.g. [21,22]). Studies of the advantages of sperm gigantism, the trade-off of sperm quality against quantity and the coevolution of traits related to reproduction with giant sperm in organisms other than ostracods are manifold (e.g. [23]). In Ostracoda, giant sperm are considered to have a single origin and are a characteristic trait of the entire suborder Cypridocopina [13]. A previous record of preserved female seminal receptacles, as well as male claspers and Zenker organ tubes, in a phosphatized cypridoidean ostracod from the Santana Formation of Brazil (approximately 110 Ma [24]) implied that these ostracods possessed giant sperm and that the origin of this reproductive mode was even older [2,4]. However, only the axial tubes of the Zenker organs were found and no sperm were evident, although their presence was inferred from the filled shapes of the female seminal receptacles [4]. The giant sperm and Zenker organ morphology in our amber specimens therefore provide the first direct evidence that this remarkable reproductive mode already existed in the Early Cretaceous.

The sperm in our amber specimens represent the oldest fossil record of ostracod sperm, predating (by approximately 83 million years) those in phosphatized Miocene ostracods from Riversleigh, Australia, which were hitherto considered the oldest [5]. Insect sperm of Cretaceous age has been reported in a mole cricket entombed in mid-Cretaceous amber from Myanmar that was described as having a spermatophore, with a single sperm partly extruded, attached to its abdomen [25]. The published figure, however, is inconclusive, and we consider it likely that the feature is actually a long thin 'sperm tube', which is characteristic of the spermatophore in this group [26]. Our material is approximately 50 Myr older than the two fossil records of animal sperm previously considered the oldest, these being isolated and bundled sperm from unknown producers in amber from Corbières, France [27], and sperm embedded in the matrix of an Antarctic clitellate annelid cocoon [28]. We therefore consider the giant sperm in the seminal receptacles of our specimens to be by far the oldest animal sperm of any kind yet identified.

Our discovery highlights the capacity of amber to document invertebrate soft parts that are rarely recorded by other depositional environments. The male clasper, sperm pumps, hemipenes and female seminal receptacles with giant sperm of fossil ostracods reveal that the reproduction behavioural repertoire, which is associated with considerable morphological adaptations, has remained unchanged over at least 100 million years. The appearance of a complex reproductive mechanism involving giant sperm improves mating success and may have been an important contributor to the late Mesozoic explosive radiation of the superfamily Cypridoidea [29], which today includes the vast majority of nonmarine ostracod species.

4. Materials and methods

The studied amber piece came from an amber mine near Noiye Bum Village, Danai Town in northern Myanmar. The U-Pb dating of zircons from the volcanoclastic matrix of the amber gave a maximum age of 98.8 ± 0.6 million years [30], which is also supported by the ammonite trapped in the amber [7]. The studied amber piece is 17.5 mm long, 13.5 mm wide and 4.0 mm high, and its weight is 0.676 g, including two dipteran insects and 39 ostracod crustaceans (figure 1). Most of the ostracods have morphological details of the soft body and appendages preserved. Ostracod individuals are deep in the amber, lying obliquely or partly covered by mineral grains, gas bubbles or plant and insect debris, so only a few of them are accessible for light-microscopic analysis, and at low resolution (figure 1). For the same reason, not all of the ostracod specimens could be subject to micro-CT analysis.

The two flies in the amber piece belong to the fungus gnat (Mycetophilidae) genus *Protragoneura* and the biting midge (Ceratopogonidae) genus *Archiaustroconops*, respectively, that are common elements in mid-Cretaceous Kachin amber [31,32,33], confirming that the studied amber piece is Kachin amber which is late Albian–early Cenomanian in age [7]. The amber piece is deposited in the Lingpoge Amber Museum. Photographs were taken using a Zeiss AXIO Zoom V16 microscope system at the State Key Laboratory of Paleobiology and Stratigraphy, Nanjing Institute of Geology and Palaeontology, Chinese Academy of Sciences (NIGPAS). In most cases, incident and transmitted light were used simultaneously. All images are digitally stacked photomicrographic composites of approximately 40 individual focal planes that were obtained using the software Helicon Focus 6 (<http://www.heliconsoft.com>) for better illustration of the three-dimensional structures, as described by Schmidt et al. [34].

To three-dimensionally reconstruct the ostracods, we scanned the fossils at the micro-CT laboratory of NIGPAS, using a three-dimensional X-ray microscope (3D-XRM), Zeiss Xradia 520 versa. Unlike conventional micro-CT, which relies on maximum geometric magnification and a flat panel detector to achieve high resolution, three-dimensional-XRM uses CCD-based objectives to achieve higher spatial resolution. Here, we take BA19005-1 as an example, the others are in the electronic supplementary material, table S1. Based on the size of the fossil specimens, a CCD-based 20× objective was used, providing isotropic voxel sizes of $0.7185 \mu\text{m}$ with the help of geometric magnification. During the scan, the acceleration voltage for the X-ray source was 60 kV (power 5 W), and a thin filter (LE1) was used to avoid beam-hardening artefacts. To improve signal-to-noise ratio, 3401 projections over 360° were collected, and the exposure time for each projection was 9 s. Volume data processing was performed using software VGStudio Max (version 3.0; Volume Graphics) and the open-source volume exploration tool Drishti v2.6 [35]. Scanning electron microscopy was carried out on gold-coated specimens at 15 kV (Polaron SputterCoater; Leo 1430VP).

We analysed 11 ostracod specimens at the micro-CT laboratory of NIGPAS. The eight specimens shown in this study have complete carapaces with soft parts preserved (figure 2). Generally, the first and second pairs of antennae are the most frequently observable appendages. Most specimens are preserved with mandibula and uropodal ramus. The other appendages, like maxillula and thoracic legs, are only partly preserved in some ostracod specimens. Furthermore, the specimen BA19005-1 displays well-preserved Zenker organs and sperm duct, and the specimen BA19005-2 preserves sperm and eggs (figure 3).

Data accessibility. The amber is deposited in the Lingpoge Amber Museum (No. 59 Building, No. 2338 Duhui Road, Minhang District, Shanghai 201108, China) permanently, in full compliance with the International Code of Zoological Nomenclature and the instructions of the International Palaeoentomological Society [36,37]. The fossils in the Lingpoge Amber Museum are held safely in trust for the benefit of researchers and educators in the world respecting all ethnic groups, ages, sexes, landowners and collectors. Apart from public

exhibitions, access is free to all scientists and interested people by prior arrangement during normal working hours and subject to laboratory and museum regulations. All data needed to evaluate the conclusions in the paper are present in the paper and/or the electronic supplementary material. Additional data related to the paper may be requested from the authors (H.W.). All data related to the paper are also deposited in the Geobiodiversity Database (GBDB, <http://www.geobiodiversity.com>). Correspondence and requests should be addressed to H.W. or B.W.

Authors' contributions. H.W. and B.W. designed the project. H.W., B.W., R.M.-K. and D.J.H. wrote the manuscript. H.W., B.W., R.M.-K., D.J.H. and M.C. performed the comparative and analytical work. H.W., R.M.-K. and X.Z. collected data and prepared the figures. M.C. and H.Z. collected data and contributed to the discussion.

Competing interests. The authors declare no competing interests.

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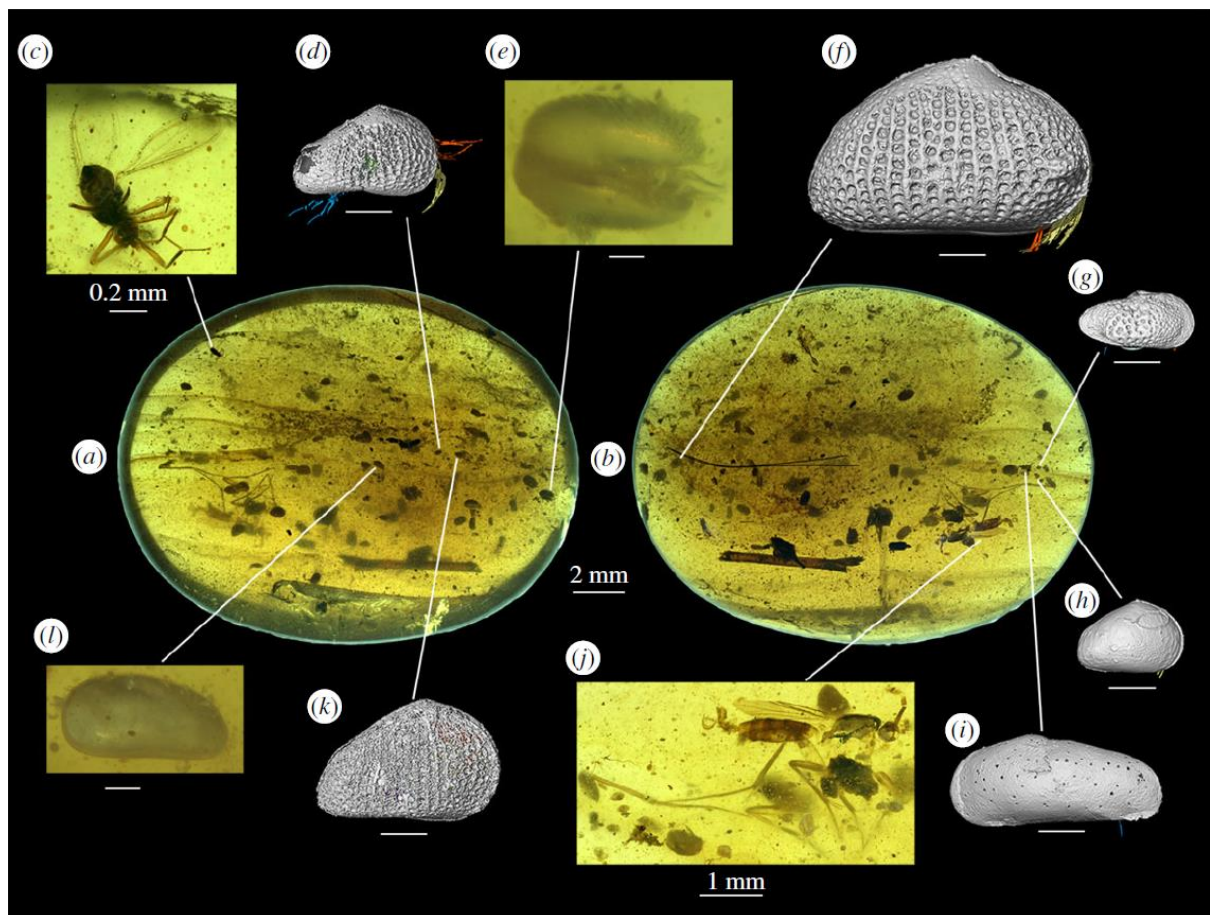


Figure 1. The studied amber piece showing the position of the studied specimens (BA19005). (a,b) Different sides of the studied amber piece. (c) *Atriculicoides* (Diptera: Ceratopogonidae), BA19005-9. (d) Specimen BA19005-4. (e) Specimen BA19005-1. (f) Specimen BA19005-2. (g) Specimen BA19005-8. (h) Specimen BA19005-5. (i) Specimen BA19005-6. (j) *Protragoneura* (Diptera: Mycetophilidae), BA19005-10. (k) Specimen BA19005-3. (l) Specimen BA19005-7. Scale bar = 100 μ m. (Online version in colour.)

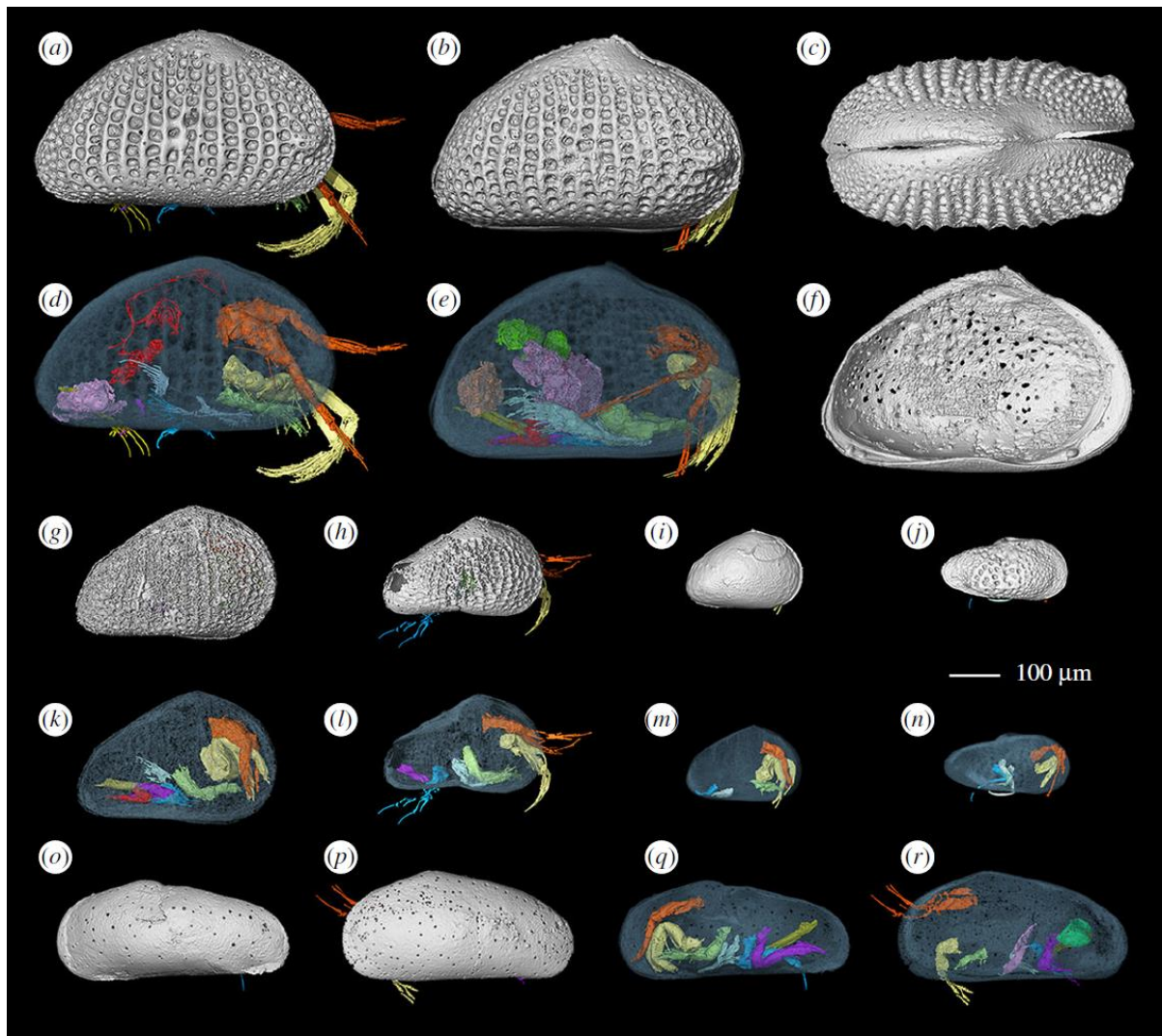


Figure 2. The studied ostracods from Myanmar amber: microtomographic reconstructions. (a,b) *Myanmarocypris hui* gen. et sp. nov., surface rendering, right view. (a) Male, BA19005-1. (b) Female, BA19005-2. (c) Dorsal view of adult female carapace, surface rendering, BA19005-2. (d,e) *Myanmarocypris hui* gen. et sp. nov., surface rendering with transparency, right view. (d) Male, BA19005-1. (e) Female, BA19005-2. (f) Internal view of adult female left valve, surface rendering, BA19005-2. (g–i) *Myanmarocypris hui* gen. et sp. nov., juveniles, surface rendering, right view. (g) Juvenile (A-2), BA19005-3. (h) Juvenile (A-3), BA19005-4. (i) Juvenile (A-4), BA19005-5. (j) *Sanyuania* sp., BA19005-8, surface rendering, right view. (k–m) *Myanmarocypris hui* gen. et sp. nov., juveniles, surface rendering with transparency, right view. (k) Juvenile (A-2), BA19005-3. (l) Juvenile (A-3), BA19005-4. (m) Juvenile (A-4), BA19005-5. (n) *Sanyuania* sp., BA19005-8, surface rendering with transparency, right view. (o,p) *Thalassocypris* sp., surface rendering, left view. (o) BA19005-6. (p) BA19005-7. (q,r) *Thalassocypris* sp., surface rendering with transparency, left view. (q) BA19005-6. (r) BA19005-7. Note: colours for the appendages and soft parts correspond to figure 3 (in which they are labelled). (Online version in colour.)

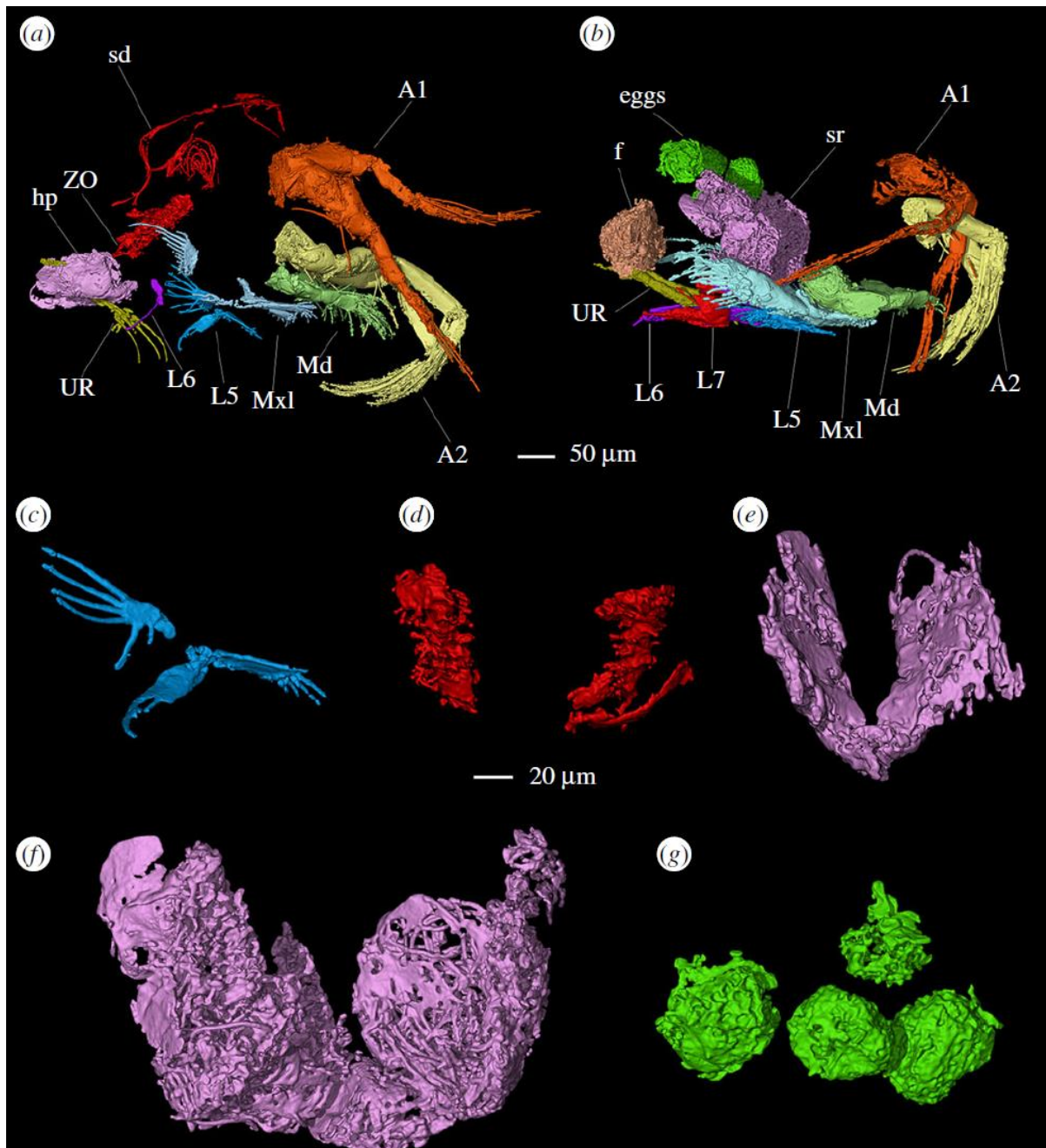


Figure 3. Soft parts of *Myanmarocypris hui* gen. et sp. nov. with illustration, surface rendering. (a) Male, BA19005-1. (b) Female, BA19005-2. (c) Right L5 with clasper. (d) Zenker organs. (e) Hemipenes. (f) Seminal receptacles (filled with giant sperms). (g) Eggs. A1, first antenna (antennula); A2, second antenna (antenna); Md, mandibula; Mxl, maxillula; L5–L7, fifth to seventh limbs; UR, uropodal ramus; ZO, Zenker organ; hp, hemipenes; sd, sperm duct (coiled); sr, seminal receptacle; f, probably faeces. (Online version in colour.)

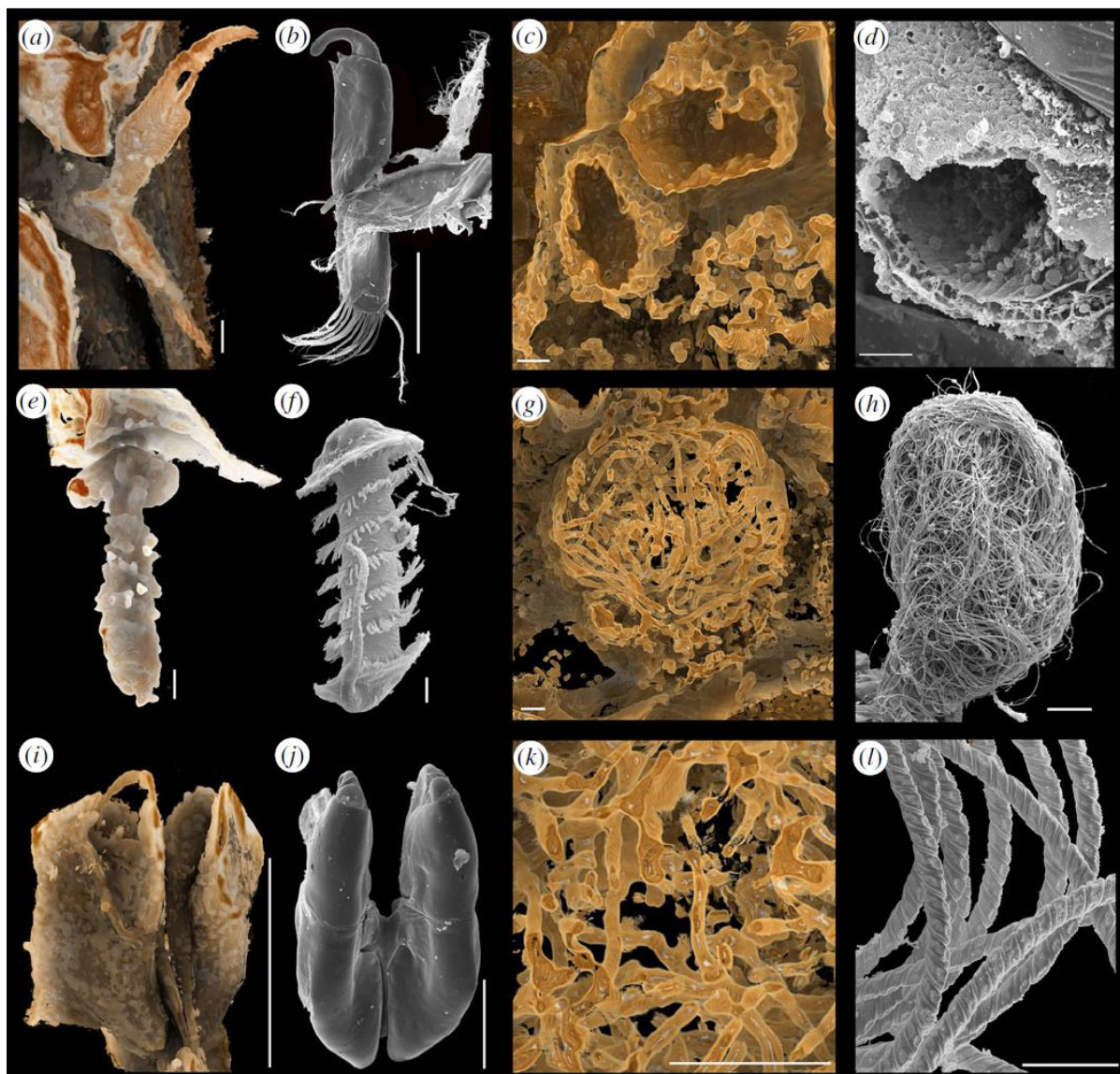


Figure 4. Preserved body parts in fossils in comparison with modern analogues. Body parts of *Myanmarocypris hui* gen. et sp. nov. were visualized by tomography and volume rendering of female (BA19005-2) and male (BA19005-1) adult individuals, modern analogues by scanning electron microscopy. (a) Right 5th limb of male with clasping organ (top). (b) *Eucypris virens*: right 5th limb of male with clasping organ (top). (c) Two eggs in female partly cut open. (d) *E. virens*: empty egg shell. (e) Zenker organ. (f) *Cyclocypris ovum*: Zenker organ. (g) Mass of sperms in location of seminal receptacle in female. (h) *Mytilocypris mytiloides*: densely packed sperm filling of a seminal receptacle. (i) Paired hemipenes. (j) *Fabaeformiscandona subacuta*: paired hemipenes. (k) Sperms in female. (l) *Pseudocandona marchica*: sperms in female. Scale bars, 10 μ m (a), (c–g), (k), (l); 100 μ m (b), (h–j). (Online version in colour.)



Figure 5. Artist's reconstruction of *Myanmarocypris hui* in life (a mating pair). (Online version in colour.)